

*Commercial Vegetative Inoculum of *Pisolithus tinctorius* and Inoculation Techniques for Development of Ectomycorrhizae on Container-grown Tree Seedlings*

D. H. MARX, J. L. RUEHLE,
D. S. KENNEY, C. E. CORDELL,
J. W. RIFFLE, R. J. MOLINA,
W. H. PAWUK, S. NAVRATIL,
R. W. TINUS, and O. C. GOODWIN

ABSTRACT. Vegetative inoculum of *Pisolithus tinctorius* produced by research procedures at the Institute for Mycorrhizal Research and Development (IMRD) was compared with that produced in large commercial solid substrate fermentors by Abbott Laboratories. Effectiveness of *P. tinctorius* in forming ectomycorrhizae on container-grown seedlings from this inocula was

The authors are D. H. Marx, Chief Plant Pathologist and Director, and J. L. Ruehle, Plant Pathologist, USDA Forest Service, Institute for Mycorrhizal Research and Development, Forestry Sciences Laboratory, Athens, Georgia; D. S. Kenney, Section Head, Microbial Products Research, Abbott Laboratories, North Chicago, Illinois; C. E. Cordell, Plant Pathologist and National Mycorrhizae Coordinator, USDA Forest Service, Forest Insect and Disease Management, Southeastern Area, State and Private Forestry, Asheville, North Carolina; J. W. Riffle, Plant Pathologist, USDA Forest Service, Forestry Sciences Laboratory, Lincoln, Nebraska; R. J. Molina, Botanist, USDA Forest Service, Forestry Sciences Laboratory, Corvallis, Oregon; W. H. Pawuk, Plant Pathologist, USDA Forest Service, Forestry Sciences Laboratory, Pineville, Louisiana (present address, Tongass National Forest, Petersburg, Alaska); S. Navratil, Professor, School of Forestry, Lakehead University, Thunder Bay, Ontario, Canada; R. W. Tinus, Plant Physiologist, USDA Forest Service, Shelterbelt Laboratory, Bottineau, North Dakota; and D. C. Goodwin, Senior Project Forester, North Carolina Division of Forest Resources, Raleigh, North Carolina. Manuscript received 11 March 1981.

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observed on 10 pine species, Douglas-fir, western hemlock, and bur oak in tests at six locations in the United States and one in Canada. Inoculum was mixed into rooting media before sowing of seed. The Abbott inoculum (Mycorhiz[®]) was ineffective on pine seedlings in tests of 1977, but changes in fermentation procedures significantly improved inoculum effectiveness in 1978 and 1980 to such a degree that certain batches of Mycorhiz[®] were as effective as IMRD inoculum.

A medium of vermiculite and 5 to 10 percent by volume peat moss with nutrient was best for growing mycelial inoculum. Peat moss, which contains humic acids, was used for keeping pH of inoculum below 6.0 at which range it was the most effective. Inoculum was most effective after leaching with water to remove nutrients. No one inoculum characteristic, such as number of *P. tinctorius* propagules in large and small particles, microbial contamination, residual glucose, bulk density, and moisture content, as well as results of a fast assay for ectomycorrhizal development on loblolly pine seedlings, was consistently correlated with effectiveness of inoculum in forming *P. tinctorius* ectomycorrhizae on seedlings in containers. A captan drench after seeding significantly improved effectiveness of inoculum that was initially low in effectiveness.

Seedling growth was correlated with *P. tinctorius* ectomycorrhizal development in only a few tests. The probable cause for lack of growth stimulation of seedlings by *P. tinctorius* ectomycorrhizae is a photosynthate drain on the juvenile seedlings by *P. tinctorius*. Jack pine seedlings grown in a medium containing high levels of N, P, and K formed about half as many *P. tinctorius* ectomycorrhizae as similarly treated seedlings grown at about half of this fertility. The seedlings grown at high fertility were, however, larger regardless of ectomycorrhizal treatment.

Results of the tests in 1978 and 1980 showed that viable vegetative inoculum of *P. tinctorius* in a substrate of vermiculite, peat moss, and nutrient can be produced by industrial fermentation procedures and used to form abundant *P. tinctorius* ectomycorrhizae on container-grown seedlings for practical use in forest regeneration. *FOREST SCI.* 28:373-400.

ADDITIONAL KEY WORDS. *Pinus taeda*, *Pinus echinata*, *Pinus palustris*, *Pinus elliotii* var. *elliotii*, *Pinus virginiana*, *Pinus ponderosa*, *Pinus sylvestris*, *Pinus clausa*, *Pinus nigra*, *Pinus banksiana*, *Pseudotsuga menziesii*, *Tsuga heterophylla*, *Quercus macrocarpa*, *Thelephora terrestris*, *Pisolithus tinctorius* (Pt) index, seedling quality.

MANY SPECIES OF TREES require ectomycorrhizal fungus associations on their feeder roots for normal growth and development in a natural forest environment. Various methods have been devised to ensure that ectomycorrhizae are formed on nursery seedlings. Forest soil or humus, excised ectomycorrhizae, crushed sporophores of selected fungi, or spores have been added as inoculum to nursery soil before or shortly after sowing seed. If the procedures were followed properly, these methods usually produced abundant ectomycorrhizae on seedlings. Various reviewers of past work (Bowen 1965, Mikola 1973, Trappe 1977, Marx 1980) agree that the use of pure mycelial cultures of selected fungi is the most biologically sound inoculum method since harmful organisms are excluded.

Beginning in the early 1950's, Moser in Austria pioneered the use of pure fungus cultures as inoculum (Marx 1980). Initially, Moser (1958) developed techniques to form ectomycorrhizae on seedlings of *Pinus cembra* with strains of *Suillus plorans* that grew well at low temperatures in order to improve seedling performance on cold sites near timberline in the Alps. Mycelial inoculum of *S. plorans* was grown in small containers of liquid nutrient, then transferred to 10-l tanks of nutrients for 3 to 4 months of incubation. This mycelium was used to inoculate 5-l flasks containing sterile peat moss. After a few months of growth, flask contents were used as inoculum. Moser tried several inoculation procedures in the nursery and obtained best results with 8- to 10-l inoculum/m² of soil surface, mixed 10 cm deep into the soil prior to transplanting young seedlings. Moser found that inoculum was most effective in soil previously heated or treated with formalin.

Takacs (1967) in Argentina, Theodorou and Bowen (1970) in Australia, and Vozzo and Hacskeylo (1971) in the United States modified Moser's techniques by substituting vermiculite or cereal grain for peat moss and successfully formed

ectomycorrhizae on pine seedlings with pure cultures of selected fungi. In all these studies, field survival and growth of seedlings with specific ectomycorrhizae exceeded the performance of seedlings that lacked ectomycorrhizae at planting.

Moser's concept of forming ectomycorrhizae on tree seedlings in nurseries with fungi ecologically adapted to the planting site was explored in the southern United States by the U.S. Forest Service. This was prompted by the comprehensive report of Schramm (1966) on plant colonization of anthracite coal wastes in Pennsylvania, and observations by him and others (Lampky and Peterson 1963, Meyer 1968, Hile and Hennen 1969, Marx 1977a, Medve and others 1977) that sporophores and ectomycorrhizae formed by the fungus *Pisolithus tinctorius* (Pers.) Coker and Couch are found in great abundance on various tree species growing on coal and kaolin spoils, borrow pits, and other adverse sites. These sites are normally characterized by high soil temperatures during summer, extreme acidity, droughtiness, low fertility, or high levels of toxic metals. Usually, seedlings with *P. tinctorius* ectomycorrhizae were the most vigorous seedlings found on these sites and its yellow-gold ectomycorrhizae were the first detected on roots of volunteer seedlings. Laboratory and growth room studies helped to explain the persistence of *P. tinctorius* on adverse sites. In pure culture, the fungus was capable of growing at 40° to 42°C and grew most rapidly at 28° to 30°C (Marx and others 1970, Momoh and Gbadegesin 1980, Hung and Chien 1978). The thermal death point of *P. tinctorius* hyphae was 45°C (Lamb and Richards 1971). Other ectomycorrhizal fungi tested had much lower temperature tolerances and optima. Under aseptic conditions, loblolly pine (*Pinus taeda* L.) seedlings formed much more ectomycorrhizae with *P. tinctorius* at 34°C than at lower temperatures (Marx and others 1970). Also, aseptically grown loblolly pine seedlings ectomycorrhizal with *P. tinctorius* had better survival and growth at 40°C in laboratory tests than nonmycorrhizal seedlings or those ectomycorrhizal with *Thelephora terrestris* (Ehrh.) Fr. (Marx and Bryan 1971).

Pisolithus tinctorius can thus be a biological tool to improve survival and growth of pines on both poor quality forestation sites and reclamation sites. It has several characteristics which make it suitable for practical application. This fungus can easily be propagated in the laboratory on a variety of solid or liquid media. The yellow-gold or mustard color of its hyphae facilitates detection and quantitative assessment of ectomycorrhizae on seedling roots. It also produces abundant hyphal strands in pure culture and on seedling roots. Hyphal strands develop as extramatrical growth from roots into the surrounding soil and, apparently, increase the nutrient (Bowen 1973) and water (Duddridge and others 1980) absorbing efficiency of ectomycorrhizal fungi. Sporophores of *P. tinctorius* are readily identified without microscopic examination because brown-yellow spores produced in compartments (peridioles) are a unique characteristic. This fungus has a proven host range of over 50 tree species and, under field conditions, has been associated with an additional 25 tree species; it has been reported from over 33 countries of the world and 38 states in the United States (Marx 1977b). In addition to adverse sites, it is found in urban areas, orchards, many forest sites, and occasionally in tree nurseries (Grand 1976, Marx 1977b, Malloch and Kuja 1979). Since this fungus is ecologically adapted to adverse soil conditions which characterize most reclamation sites and many temporarily adverse reforestation sites (Schultz 1979), pine seedlings with *P. tinctorius* ectomycorrhizae often have a survival and growth advantage over seedlings with ectomycorrhizae formed by other fungi that occur naturally in nurseries.

One of the most common fungi occurring naturally in forest nurseries throughout the world is *Thelephora terrestris* (Weir 1921, Hacskeylo 1965, Mikola 1973). It is ecologically adapted to the excellent tilth, fertility, and moisture conditions of nursery soil but it often fails to adapt to the harsh soil conditions of many

outplanting sites (Marx and Artman 1979, Marx 1977a). Because of adaptation to nursery soil environment, *T. terrestris* and other naturally occurring fungi are major competitors of introduced pure culture inoculum of select fungi for seedling roots.

Marx (1980) discussed in detail the early testing and development stages of producing viable inoculum of *P. tinctorius* for research purposes. The basic procedure involves growing mycelium of the fungus for 3 or 4 months at room temperature in 1.5-l jars containing a mixture of vermiculite and peat moss moistened with modified Melin-Norkrans (MMN) liquid medium containing glucose. The substrate permeated by the fungus is removed from the jars, leached in tap water, and dried for 60–90 hrs at 25° to 30°C to a moisture content of 12 to 20 percent based on oven-dry weight (Marx and Rowan 1981). Dried inoculum has been stored in small quantities, without losing significant viability, for 5 wks at room temperature and up to 9 wks at 5°C. Mycelium which develops in the laminated structure of the vermiculite particles maintains structural integrity and is protected from environmental stress and microbial saprophytic competition. Mycelium of this fungus grown in other substrates, such as sand, perlite, peat moss, and cereal grains, has little or no protection from these factors.

Vermiculite-based inoculum has been used successfully to form *P. tinctorius* ectomycorrhizae in fumigated soil on pine, oak, and pecan seedlings in experimental microplots (Marx and Bryan 1975, Krugner 1976, Marx 1979a,b,c) and on pine seedlings in conventional bare-root nurseries in Georgia, Florida, and North Carolina (Marx and others 1976), Virginia (Marx and Artman 1978), Oklahoma (Marx and others 1978), and Mississippi (Marx 1980). In most of these nursery tests, seedlings with abundant *P. tinctorius* ectomycorrhizae grew larger than control seedlings with naturally occurring ectomycorrhizae. Fumigation of nursery soil prior to inoculation improves ectomycorrhizal development because it lowers populations of (1) soil microorganisms that can colonize introduced inocula (Bowen and Theodorou 1979, Marx 1980); (2) feeder root pathogens that damage roots and thus reduce ectomycorrhizal development (Ruehle 1973, Marx and others 1976); and (3) indigenous competing ectomycorrhizal fungi such as *T. terrestris* from previous tree crops (Marx and others 1976, 1978). Vermiculite-based inoculum broadcast at a rate of about 1 l/m² of soil surface and mixed in soil has been as effective as higher rates in forming abundant *P. tinctorius* ectomycorrhizae (Marx 1980).

Vermiculite-based inoculum also has been used successfully in forming *P. tinctorius* ectomycorrhizae on container-grown tree seedlings in various media and container types (Marx and Barnett 1974, Marx 1975, Ruehle and Marx 1977, Molina 1979, Dixon and others 1979, Maronek and Hendrix 1980, Pawuk and others 1980, Ruehle and others 1981). These authors found that fertility, type of container, growing medium, fungicides, inoculum storage, and frequency of watering all influence effectiveness of the inoculum.

The value of *P. tinctorius* ectomycorrhizae to tree seedlings was indicated by the performance of seedlings under diverse field conditions. Dramatic improvements in survival and growth of pine seedlings with abundant *P. tinctorius* ectomycorrhizae over naturally infected control seedlings produced in containers or bare-root nurseries have been reported from studies on acid coal spoils in Appalachia (Marx 1977a, Marx and Artman 1979, Walker and others 1980), kaolin spoils in Georgia (Marx 1977a, Otrosina 1977), severely eroded sites of the Copper Basin in Tennessee (Berry and Marx 1978), borrow pits in South Carolina (Ruehle 1980) and North Carolina (Goodwin 1980), and prairie soil (Baer and Otta 1981). Similar improvements in pine seedling performance were reported on routine reforestation sites in Florida and North Carolina (Marx and others 1977a), Oklahoma and Arkansas (Mexal 1980, Ruehle and others 1980), Georgia (Marx 1979d),

Mississippi (Kais and others 1981), and with seedlings of *Pinus caribaea* planted on afforestation sites in Nigeria (Momoh and Gbadegesin 1980) and Liberia (Marx, unpublished data). However, on poor soils amended with fertilizer or sewage sludge, pine seedlings with *T. terrestris* ectomycorrhizae have survived and grown as well as those with abundant *P. tinctorius* ectomycorrhizae (Marx and others 1977a, Berry and Marx 1980). To obtain maximum growth of seedlings of southern pines with *P. tinctorius* ectomycorrhizae that have been planted on reforestation sites, a threshold level of at least half of all ectomycorrhizae must be by *P. tinctorius* at planting (Marx and others 1977a, Kais and others 1981, Ruehle and others 1981, Ruehle and Brendemuehl 1981). Frequently, pine seedlings with less than half of all ectomycorrhizae formed by *P. tinctorius* grew at the same rate as seedlings with the same amount of *T. terrestris* ectomycorrhizae on routine forestation sites.

The use of *P. tinctorius* on a broad scale has been prevented by lack of sufficient quantities of commercially available vegetative inoculum. In 1976, the Institute for Mycorrhizal Research and Development (IMRD) and Abbott Laboratories, North Chicago, Illinois, began a cooperative research program to develop commercial methods for producing vermiculite-based vegetative inoculum of *P. tinctorius* and to test the inoculum under diverse cultural conditions.

Because different isolates of *P. tinctorius* vary in mycelial growth and in ability to form ectomycorrhizae (Molina 1979, Marx 1981), a single isolate of *P. tinctorius* able to form abundant ectomycorrhizae on a variety of tree hosts under different conditions was used throughout this investigation. This isolate was originally obtained from a sporophore found under mature loblolly pines in Clarke County, Georgia, in 1967. Its vitality was maintained by inoculation of roots and reisolation from ectomycorrhizae formed on loblolly pine at 1- to 3-year intervals (Marx 1981). This isolate has been widely used in the United States and abroad in numerous ectomycorrhizal studies. Designations of this isolate were 155, 227, 246, and 250 for 1977, 1978, 1979, and 1980, respectively.

This paper reports a chronological series of studies that led to techniques for commercial production of pure culture inoculum and for inoculation of container-grown tree seedlings with *Pisolithus tinctorius*. Vegetative inoculum of *P. tinctorius* produced with different commercial fermentation equipment and with various procedures was tested on container-grown tree seedlings to determine the effect of this inoculum on development of *P. tinctorius* ectomycorrhizae and on seedling growth. In all tests, vegetative inoculum produced at the IMRD was used as a standard for comparison. These tests were performed on a variety of tree species in several different locations employing highly diverse cultural conditions. This was done to determine which factors were significant to the formation of ectomycorrhizae by *P. tinctorius* from the various inoculum sources.

1977 TESTS

MATERIALS AND METHODS

The following experiments were installed during March and April 1977 at the IMRD, and in North Carolina and North Dakota. IMRD inoculum was grown in vermiculite-peat moss-MMN nutrient for 3 to 4 months, leached (Marx and Bryan 1975), and dried (Marx and Rowan 1981) to a bulk density of 342 g/l with a moisture content of 42 percent.

The 1977 Abbott inoculum was produced in a vertical, deep tank, solid substrate fermentor. The substrate contained vermiculite and MMN liquid medium with slightly higher than normal amounts of carbohydrate and organic and inorganic nitrogen. The fermentor filled with the substrate was steamed (72° to 84°C), cooled, and inoculated with mycelium of *P. tinctorius*. Starter mycelium had

been grown in a deep tank, aerated, submerged culture containing the same liquid medium. The culture was incubated in the fermentor, and the inoculum was dried to a bulk density of 288 g/l with a moisture content of 21 percent. The inoculum was not leached. Abbott inoculum was shipped in drums from Chicago, Illinois, to the IMRD. Inoculum from both sources was packaged under nonsterile conditions at the IMRD in polyethylene bags in volumes sufficient to accommodate specific treatments and stored at 5°C until use or shipment. Inoculum was shipped to North Carolina and North Dakota in polyethylene ice chests containing artificial ice in plastic containers to keep the inoculum cool in transit. Inoculum from both sources was utilized at the three test locations within 18 days after drying. At all locations, IMRD inoculum was mixed with growing medium at 6 percent by volume and Abbott inoculum was mixed at 3, 6, and 12 percent by volume. Controls received no inoculum. Inoculum medium with or without killed *P. tinctorius* was not added to growing medium of control seedling because with IMRD inoculum earlier results (unpublished) showed that it did not affect pine seedling growth or development of naturally occurring ectomycorrhizae. Two pine species were tested at each location in an experimental design with five inoculum treatments replicated four times (container units) in randomized blocks.

At the IMRD, inoculum thoroughly mixed with milled pine bark (Ruehle and Marx 1977) was placed in Hillson Roottrainers®. Thirty-two seedling cavities (each 125 cc volume) placed in a rack comprised a treatment replication. Seeds of Choc-tawhatchee sand pine (*Pinus clausa* (Chapm. ex Engelm.) Vasey ex Sarg. var. *immarginata* D. B. Ward) and slash pine (*P. elliottii* Engelm. var. *elliottii*) were stratified, coated with Arasan® and latex sticker, and planted into cavities of 20 racks per tree species. After germination, seedlings were thinned to one per cavity. Three wks after seed germination and every 3 wks thereafter, 30 ml of water-soluble NPK fertilizer (23:19:17 formulation) diluted to 2,500 mg/l were added to each cavity. Six wks after germination, 30 ml of 5 percent Sequestrene 330-Fe® (10 percent chelated iron) were added to each cavity to correct an iron deficiency. Greenhouse temperatures ranged from 22° to 30°C. Seedlings were watered as needed to prevent drying, usually three times weekly. Day length varied from 12 to 15 hrs and light quantity was approximately 75 percent of full sunlight. The study was terminated 16 wks after seeding.

In North Carolina, inoculum was mixed with a 3:1 volume ratio of peat moss and horticultural grade vermiculite containing sufficient dolomitic limestone to obtain pH 4.6. Ferdinand Roottrainers® were filled with the mixture. Forty-eight seedling cavities (each 40 cc volume) placed in a rack comprised a treatment replication. Nontreated seeds of loblolly pine or Virginia pine (*P. virginiana* Mill.) were planted into cavities of 20 test racks/tree species. A 15-sec mist spray was applied at 10-min intervals for 8 hrs daily for 2 wks to hasten germination, after which seedlings were thinned to one per cavity. Water-soluble fertilizer (NPK) was applied to saturation of growing medium at the following frequencies and rates: once at 2 wks with 15-4-22 mg/l; once at 3, 4, and 5 wks with 150-42-122 mg/l; once at 7 wks with 15-100-33 mg/l; and once at 9 wks with 0-0-83 mg/l. Greenhouse temperatures ranged from 18° to 38°C. Day length averaged 14 hrs and light quantity was approximately full sunlight. The study was terminated 12 wks after seeding.

In North Dakota, inoculum mixed with 1:1 volume ratio of horticultural grade #2 peat moss and vermiculite was placed in modified Tinus Roottrainers®. Forty seedling cavities (each 410 cc volume) placed in a rack comprised a treatment replication. Nonstratified seeds of ponderosa pine (*P. ponderosa* Dougl. ex Laws.) and Scotch pine (*P. sylvestris* L.) were sterilized in 30 percent H₂O₂ for 30 and 10 mins, respectively. Seeds were planted and covered with a 5-mm layer

of perlite. The 40 racks comprising the test were lightly watered daily for 21 days or until germination was complete. Seedlings were thinned to one per cavity. Each time irrigation was required the seedlings were watered until saturation of medium with a nutrient solution containing N, P, and K at 20, 31, and 155 mg/l, respectively, at pH 5.5 (Tinus and McDonald 1979). Greenhouse temperatures ranged from 16° to 31°C. In addition to normal sunlight, seedlings received 86 watts/m² of extra incandescent lighting for 12 secs of every 6 mins throughout the night. This was done to interrupt the dark period and induce more vegetative growth. After 20 wks, the seedlings were hardened-off in the greenhouse for eventual outplanting by reducing day and night temperature settings 5°C and turning off lights at night for an additional 6 wks, after which the study was terminated.

To obtain a midstudy estimate of inoculum effectiveness five seedlings per treatment replicate in the North Dakota study were removed after 13 wks for root assessments. These randomly selected seedlings were packaged and shipped to the IMRD, as described below, for evaluation of ectomycorrhizae.

At the termination of each study, 10 seedlings per treatment replicate were randomly removed from the containers and their roots washed carefully to remove the growing medium. At North Carolina and North Dakota, seedlings were wrapped in wet paper towels, placed in treatment-labeled plastic bags, packed in ice chests with artificial ice, and shipped to the IMRD for assessment. Upon arrival, seedling heights and top and root fresh weights were measured. Roots were visually assessed without magnification (Marx and Bryan 1975) for ectomycorrhizal development. Incidence of sporophores of *P. tinctorius* was recorded during the course of each study and those of *T. terrestris* were recorded from sample seedlings at the IMRD.

Data on ectomycorrhizae were integrated into a *Pisolithus tinctorius* (Pt) index using the formula $a \times (b/c)$, where a = percent of assessed seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for seedlings without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi (total ectomycorrhizal development). A Pt index of 100 means that all the ectomycorrhizae formed on all the assessed seedlings were formed by *P. tinctorius*. Since field results on reforestation sites as previously described indicated that pine seedlings obtain significant benefit from *P. tinctorius* only when at least half of all ectomycorrhizae on their roots at planting were formed by that fungus, a Pt index of 50 was considered to be the lowest desirable value for effective inoculum. An index of 50 indicated that the majority of seedlings had their ectomycorrhizal root systems dominated by *P. tinctorius*. An inoculum that produced a Pt index of 90, for example, might have formed ectomycorrhizae by *P. tinctorius* on 100 percent of the seedlings (a), an average of 54 percent of short roots ectomycorrhizal with *P. tinctorius* (b), and an average of 6 percent of short roots ectomycorrhizal with other fungi, i.e., total percent of short roots ectomycorrhizal with all fungi of 60 percent (c). Substituting these values in the formula of $a \times (b/c)$, we have $100 \times (54/60) = \text{Pt index } 90$. The Pt index integrates into a single value all measurements relative to *P. tinctorius* ectomycorrhizal development which provides a sensitive measure of the viability and effectiveness of the inoculum in forming ectomycorrhizae and of the cultural conditions used during the test including competition from naturally occurring ectomycorrhizal fungi (Marx 1981).

It is important to know the percentage of seedlings with *P. tinctorius* ectomycorrhizae following inoculation and to integrate this value into the index computations. A few seedlings with abundant *P. tinctorius* ectomycorrhizae from the same treatment of seedlings with few or no *P. tinctorius* ectomycorrhizae could still yield an acceptable percentage of short roots ectomycorrhizal with *P. tinc-*

torius for the treatment average. The latter parameter, therefore, by itself is misleading. For research purposes one may be satisfied with this parameter, but from a practical forestation view only those seedlings with abundant *P. tinctorius* ectomycorrhizae could possibly respond to these ectomycorrhizae after outplanting, thereby detracting from the field significance of inoculation. The percentage of seedlings with *P. tinctorius* ectomycorrhizae has greater importance to production of container-grown seedlings than to bare-root seedlings since extramatrical spread of *P. tinctorius* from one root system to another is physically restricted in containers. This is not the case with bare-root seedlings where *P. tinctorius* spreads rapidly (Marx and others 1976).

All data were processed by analysis of variance, and significant differences among means were identified with Duncan's New Multiple Range Test at $P = 0.05$.

RESULTS AND DISCUSSION

The IMRD inoculum produced abundant *P. tinctorius* ectomycorrhizae and Pt indices above 50 on the seedlings regardless of location, pine species, or cultural conditions (Table 1). Therefore, test conditions at each of the three locations were suitable for ectomycorrhizal development by *P. tinctorius*. Sclerotia-like structures of *P. tinctorius* similar to those described by Dennis (1980) were observed in the rooting medium and attached to roots of slash and sand pines at the IMRD. The Abbott inoculum was not effective in forming ectomycorrhizae, especially at the IMRD and in North Carolina; results were somewhat better in North Dakota. In the latter location, Pt indices of IMRD inoculum were still 3 to 4 times greater than the Abbott inoculum, which in all cases failed to produce a Pt index of 50. Midstudy evaluation of pine seedlings in North Dakota revealed that naturally occurring ectomycorrhizae were infrequent compared to their occurrence on pines grown in the South for a similar period of time. Abbott inoculum significantly stimulated seedling growth, especially with the higher inoculum rates at all locations. Since growth stimulation was not related to *P. tinctorius* ectomycorrhizal development, it was suspected to be caused by either microbial contamination or residual nutrients in the nonleached Abbott inoculum.

The naturally occurring ectomycorrhizae on the pines tested at the IMRD and

TABLE 1. Results of 1977 container-grown seedling studies with vegetative inoculum of *Pisolithus tinctorius* (Pt) produced by the Institute for Mycorrhizal Research and Development (IMRD) and Abbott Laboratories. Each value is the mean of 10 seedlings from each of four replicates per treatment.¹

| Location, container, and species and Pt inoculum treatment | Height (cm) | Fresh weight (gm) | | | Percent short roots ecto- mycorrhizal with— | | Percent seed- lings with Pt | Pt index ² |
|---|----------------|----------------------|------|-------|--|--------------|--------------------------------------|--------------------------|
| | | Top | Root | Total | Pt | All fungi | | |
| IMRD:Hillson | | | | | | | | |
| Roottrainers®, slash pine | | | | | | | | |
| IMRD 6 percent | 19.2b | 5.4b | 2.2a | 7.6b | 9a | 12a | 85a | 57a |
| Abbott 12 percent | 24.5a | 6.6a | 2.3a | 8.9a | 0.3b | 11a | 18b | <1b |
| Abbott 6 percent | 21.0b | 5.3b | 2.0a | 7.3b | 0.1b | 10a | 5c | <1b |
| Abbott 3 percent | 22.1ab | 5.6b | 2.3a | 7.9b | 0.1b | 9a | 8c | <1b |
| Control | 17.8c | 4.7c | 2.1a | 6.8c | 0c | 6b | 0d | 0c |

TABLE 1. Continued.

| Location, container and species and Pt inoculum treatment | Height (cm) | Fresh weight (gm) | | | Percent short roots ecto- mycorrhizal with— | | Percent seed- lings with Pt | Pt index ² |
|--|----------------|----------------------|-------|--------|--|--------------|--------------------------------------|--------------------------|
| | | Top | Root | Total | Pt | All fungi | | |
| IMRD:Hillson | | | | | | | | |
| Roottrainers®, sand pine | | | | | | | | |
| IMRD 6 percent | 17.0b | 2.7b | 1.8b | 4.5b | 16a | 19a | 100a | 86a |
| Abbott 12 percent | 20.7a | 3.7a | 2.2a | 5.9a | 0a | 10b | 0c | 0c |
| Abbott 6 percent | 16.3b | 2.4b | 1.7b | 4.1b | 0.1b | 4c | 5b | <1b |
| Abbott 3 percent | 16.8b | 2.8b | 1.8b | 4.6b | 0b | 4c | 0c | 0c |
| Control | 16.3b | 2.4b | 1.9b | 4.3b | 0b | 5c | 0c | 0c |
| North Carolina: | | | | | | | | |
| Ferdinand Roottrainers®, loblolly pine | | | | | | | | |
| IMRD 6 percent | 13.5c | 1.0a | 0.5a | 1.5a | 50a | 63a | 100a | 76a |
| Abbott 12 percent | 14.8a | 1.1a | 0.5a | 1.6a | 3b | 20b | 20b | 4b |
| Abbott 6 percent | 14.1b | 1.0a | 0.4a | 1.4a | 2b | 36b | 16b | <1c |
| Abbott 3 percent | 13.2c | 1.0a | 0.5a | 1.5a | 2b | 28b | 13b | <1c |
| Control | 13.2c | 0.9a | 0.4a | 1.3a | 0c | 25c | 0c | 0c |
| North Carolina: | | | | | | | | |
| Ferdinand Roottrainers®, Virginia pine | | | | | | | | |
| IMRD 6 percent | 10.1b | 0.7a | 0.5a | 1.2a | 24a | 39a | 100a | 61a |
| Abbott 12 percent | 10.6b | 0.8a | 0.4a | 1.2a | 2b | 14b | 23b | 3b |
| Abbott 6 percent | 12.0a | 0.9a | 0.5a | 1.4a | 2b | 18b | 20b | 2b |
| Abbott 3 percent | 10.9b | 1.0a | 0.5a | 1.5a | 1b | 16b | 13b | <1b |
| Control | 9.7b | 0.7a | 0.5a | 1.2a | 0c | 14b | 0c | 0c |
| North Dakota: | | | | | | | | |
| Tinus Roottrainers®, Scotch pine | | | | | | | | |
| IMRD 6 percent | 11.2b | 8.4b | 8.3b | 16.7b | 23a | 34a | 100a | 63a |
| Abbott 12 percent | 14.2a | 12.2a | 13.0a | 25.2a | 9b | 35a | 65b | 17b |
| Abbott 6 percent | 12.4ab | 11.4a | 13.0a | 24.4a | 8b | 35a | 65b | 16b |
| Abbott 3 percent | 10.5b | 9.5b | 12.8a | 22.3a | 9b | 42a | 70b | 17b |
| Control | 10.6b | 8.7b | 7.6b | 16.3b | 0c | 30a | 0c | 0c |
| North Dakota: | | | | | | | | |
| Tinus Roottrainers®, ponderosa pine | | | | | | | | |
| IMRD 6 percent | 10.6b | 9.0b | 8.0ab | 17.0b | 29a | 36a | 100a | 79a |
| Abbott 12 percent | 13.4a | 12.0a | 8.8a | 20.8a | 5b | 19b | 48c | 13b |
| Abbott 6 percent | 13.1a | 11.8a | 8.6a | 20.4a | 6b | 17b | 48c | 22b |
| Abbott 3 percent | 12.8a | 10.6ab | 8.3a | 18.9ab | 9b | 22b | 70b | 31b |
| Control | 12.7a | 10.4ab | 7.3b | 17.7b | 0c | 22b | 0d | 0c |

¹ Means sharing a common letter within a location-pine species combination but between Pt inoculum treatments are not significantly different at $P = 0.05$.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

in North Carolina resembled those formed by *Thelephora terrestris* (Marx and Bryan 1971, Marx and others 1970). Some of the ectomycorrhizae on pine in North Dakota also resembled those of *T. terrestris*, but more than half of them were white ectomycorrhizae formed by unidentified fungi. Thirty-six sporophores of *P. tinctorius* were formed on ponderosa pine and 46 were formed on Scotch pine in the North Dakota test. More sporophores were formed in containers of IMRD inoculum than in containers of Abbott inoculum. Some sporophores were stalked and the rest were sessile regardless of inoculum source.

Since the 1977 Abbott inoculum was not very effective, procedures for producing and processing inoculum were modified during late 1977 and early 1978 in an attempt to improve the effectiveness of this inoculum. An expanded trial was implemented in 1978 at more locations to ascertain the effectiveness of the new inoculum.

1978 TESTS

MATERIALS AND METHODS

Experiments were installed during March and April 1978 at the IMRD and in Louisiana, Nebraska, North Dakota, Oregon, and Ontario, Canada, using the same inoculum rates and experimental design as in 1977. IMRD inoculum was produced and processed as described earlier; bulk density was 217 g/l with a moisture content of 16 percent. Unlike the inoculum produced in 1977, Abbott inoculum for the 1978 tests was produced in fermentation trays containing vermiculite and 10 percent peat moss by volume. Following steam pasteurization, the substrate was inoculated with starter mycelium, harvested, and dried, as in 1977, to a bulk density of 210 g/l with a moisture content of 28 percent. Inoculum of both sources was packaged and shipped to the test locations as previously described. All tests were installed within 21 days after drying the inoculum.

At the IMRD, the 1978 test was installed using similar materials and procedures listed for 1977 except that the container cavities were planted with treated seeds of loblolly pine and slash pine. Seedlings were thinned, fertilized, watered, and grown for 18 wks after seeding under greenhouse conditions similar to the 1977 test.

In Louisiana, inoculum mixed with a 1:1 volume ratio of the peat moss and vermiculite rooting medium was placed in cavities (each 75 ml volume) of Styroblock-4 containers having 75 cavities per replicate. Stratified seeds of shortleaf pine (*P. echinata* Mill.) and nonstratified seeds of longleaf pine (*P. palustris* Mill.) were treated with Arasan® and planted. Trays were watered daily until seed germination. Three wks after sowing, cavities were thinned to one seedling each. A water-soluble NPK fertilizer (20-19-18) at 2,500 mg/l N was applied at a 15-ml volume to each seedling at 5 and 10 wks. The greenhouse was air-conditioned to maintain about 24°C. Seedlings received full sunlight for the first 6 wks and 70 percent of full sunlight (partial shading) thereafter. Day length was 12 to 14 hrs. Seedlings were watered three times weekly. The study was terminated 16 wks after seeding.

In Nebraska, inoculum mixed with a 1:1 volume ratio of the peat moss and vermiculite rooting medium was placed in cavities (each 495-ml volume) of Colorado State Nursery Styroblock containers having 30 cavities per replicate. Seeds of ponderosa pine, Scotch pine, and Austrian pine (*P. nigra* Arnold) were soaked in 30 percent H₂O₂ for 30, 10, and 10 min, respectively, and planted. They were covered with a 1-cm layer of perlite and watered daily. Two wks after seeding, seedlings were thinned to one per cavity. Fifteen ml of a 460 mg/l solution of water-soluble NPK fertilizer (7:6:19) were added to each cavity starting 23 days after seeding and every 11 days thereafter. Greenhouse temperatures varied from

16° to 36°C during the first 23 wks of the study. During this phase, in addition to full sunlight, seedlings received 86 watts/m² of incandescent lighting for 4 min of every 30 min between 1800 and 0600 hrs each day. Seedlings were watered by demand. Seedlings were then moved to a shadehouse for 6 wks to start the hardening-off process; the study was terminated 29 wks after seeding.

In North Dakota, inoculum mixed with a 1:1 volume ratio of the horticultural grade #2 peat moss and vermiculite (3 to 5 mm particle size) rooting medium was placed in cavities (each 410 cc volume) of Tinus Roottrainers® having 40 cavities per replicate. Acorns of bur oak (*Quercus macrocarpa* Michx.) were collected, float tested, and stratified (Tinus 1978). Acorns were held at room temperature for 3 days and those with emerged radicles were planted 1 cm deep in cavities. After germination, 86 watts/m² of incandescent light were provided 12 sec out of every 6 min throughout the night to extend the 14 hr natural sunlight photoperiod. During germination, the rooting mixture was kept moist, but thereafter it was allowed to dry between irrigations. After germination, a complete, high N nutrient solution containing N, P, and K at 223, 27, and 155 mg/l, respectively, at pH 6.5 was applied every 10 days until it drained from cavities at each irrigation (Tinus 1980). Seedling hardening-off was begun by applying a low N fertilizer containing N, P, and K at 20, 60, and 155 mg/l, respectively. The greenhouse atmosphere was enriched to about 0.12 percent CO₂ whenever vents were closed during daylight hrs until seedling hardening-off began. Greenhouse temperatures were maintained at 20° to 24°C during daytime for the first 4 wks, 28° to 37°C for the next 19 wks, and then 20° to 25°C for the remaining 2 wks of the study. Night temperatures were 20° to 23°C during the first 23 wks and 15° to 17°C for the last 2 wks (hardening-off) of the study. This study was terminated 25 wks after seeding.

In Oregon, inoculum mixed with a 1:1 volume ratio of the peat moss and vermiculite rooting medium (steam at 80°C for 30 min) was placed in cavities (each 65 cc volume) of 40 Leach tubes per replicate. Stratified seeds of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) were planted and watered twice daily with a fine mist for 2 wks after which seedlings were thinned to one per cavity. Fifteen ml of a 600 mg/l solution of a water-soluble NPK fertilizer (20-19-18) and 15 ml of 2 percent Sequestrene 330-Fe® solution were applied biweekly to all cavities. Additional tap-water was added as needed. Greenhouse temperatures ranged from 16° to 28°C. In addition to full sunlight, seedlings received extra light of approximately 11,000 lx (additional 10 percent of full sunlight) from sodium-vapor lights for 15 hrs daily. The study was terminated 28 wks after seeding.

In Canada, inoculum was mixed with a 3:2 volume ratio of the peat moss and vermiculite rooting medium containing approximately 9.5 kg/m³ dolomitic limestone and 1.6 kg/m³ of monosuperphosphate. The rooting medium had a pH of 4.4. Cavities (each 40 cc volume) of Ferdinand Roottrainers® were filled with medium; 106 cavities comprised a replicate. Stratified seed of jack pine (*P. banksiana* Lamb.) were planted, covered lightly with sand, and watered twice daily. After 2 wks, seedlings were thinned to one per cavity. Three wks after thinning, two fertility levels were maintained. Seedlings in the high-fertility treatment received 12 ml each of a 2,200 mg/l solution of water-soluble NPK fertilizer (20-20-20) and 540 mg/l of NH₄NO₃ four times per wk. Those in the low-fertility treatment received 12 ml of the same solutions of both fertilizers approximately twice a wk. A single application of 12 ml/seedling of chelated iron was added to all seedlings 6 wks after germination. Both fertility treatments had four blocks each of five inoculum treatments. By the end of the study, each seedling in the high-fertility treatment had received totals of 32 mg, 10.5 mg, and 19 mg of N, P, and K, and those in the low-fertility treatment had received totals of 20 mg, 6 mg,

and 12 mg of N, P, and K, respectively. Greenhouse temperatures ranged from 17° to 30°C. Seedlings received full sunlight for the first 10 wks of the test, after which they were moved to a shadehouse to harden off. Seedlings were watered by demand. The study was terminated 14 wks after germination.

Sample seedlings from Nebraska, North Dakota, Oregon, and Canada were assessed at midstudy. These seedlings and those obtained at the termination of all studies were shipped to the IMRD where they were measured (including stem diameters) and assessed for ectomycorrhizae. Data were analyzed as previously described.

RESULTS AND DISCUSSION

Evaluations of seedlings grown in Nebraska, North Dakota, Oregon, and Canada at midstudy revealed a few naturally occurring ectomycorrhizae in comparison to seedlings grown for similar time periods in the South. Frequency of short root infections ranged from 0 to 16 percent in the northern areas at midstudy, whereas infection on southern pines ranged from 10 to 55 percent after the same period of time. Midstudy observations on relative amounts of *P. tinctorius* ectomycorrhizae developed by different inocula in the northern tests were consistent with Pt indices made at study termination.

Final results showed that the IMRD inoculum produced abundant *Pisolithus* ectomycorrhizae and, with two exceptions, produced Pt indices well above the minimum requirement on all tree species in all locations (Table 2). IMRD inoculum in the longleaf pine test in Louisiana and in the high fertility test on jack pine in Canada did not produce a Pt index of 50 or greater. The low Pt index in the Canadian test is explained by excessive soil fertility, a factor known to reduce *P. tinctorius* ectomycorrhizal development on pine (Marx and others 1977b). The low index on longleaf pine in Louisiana is probably related to the small number of lateral and short roots produced by this species in the juvenile stage of growth (Snyder 1961). If a tree species, such as longleaf pine, produces only a few short roots, the chances of these short roots growing into or otherwise affecting (via root exudates) the inoculum particles in the rooting medium and becoming ectomycorrhizal with *P. tinctorius* is much less than that of another tree species, such as shortleaf pine, which produces abundant short roots. The Pt index for the IMRD inoculum on the companion study with shortleaf pine in Louisiana exceeded the index standard, indicating that cultural conditions were adequate for ectomycorrhizal development.

At the IMRD where pine bark was used as the growing medium, Abbott inoculum formed very few *P. tinctorius* ectomycorrhizae on either pine species; whereas, IMRD inoculum was highly effective. On shortleaf pine in Louisiana where peat moss and vermiculite was the growing medium, the Abbott inoculum in the 12 percent volume treatment produced a Pt index of 65 which was as high as the IMRD inoculum index. Like the IMRD inoculum, the Abbott inoculum was relatively ineffective on longleaf pine in Louisiana.

In the Nebraska (three pines), North Dakota (bur oak), and Oregon (fir and hemlock) tests, Abbott inoculum produced consistently more *P. tinctorius* ectomycorrhizae than it did with the southern pines at other locations. In these states the growing medium was peat moss and vermiculite, and the growth period was several wks longer. In these tests, the Pt indices for the 12 and 6 percent rates of Abbott inoculum exceeded the minimum index of 50 in all but one treatment (6 percent Abbott inoculum on Douglas-fir). Both IMRD and Abbott inoculum were generally less effective on western hemlock than on Douglas-fir in Oregon. On bur oak seedlings the Pt indices for Abbott inoculum at all rates were no different than IMRD inoculum.

The results in Canada showed the inhibitory effects of high fertility on ecto-

TABLE 2. Results of 1978 container-grown seedling studies with vegetative inoculum of *Pisolithus tinctorius* (Pt) produced by the Institute for Mycorrhizal Research and Development (IMRD) and Abbott Laboratories. Each value is the mean of 10 seedlings from each of four replicates per treatment.¹

| Location, container, and species and Pt inoculum treatment | Height (cm) | Stem diam (mm) | Fresh weight (gm) | | | Percent short roots ectomycor- rhizal with— | | Percent of seed- lings with Pt ecto- mycor- rhizae | |
|---|----------------|----------------------|-------------------|-------|-------|--|--------------|--|------|
| | | | Top | Root | Total | Pt | All fungi | Pt index ² | |
| | | | | | | | | | |
| IMRD:Hillson Roottrainers®, loblolly pine | | | | | | | | | |
| IMRD 6 percent | 11.9a | 2.0bc | 2.0bc | 1.3a | 3.3a | 52a | 58a | 100a | 89a |
| Abbott 12 percent | 12.4a | 2.2a | 2.4a | 1.3a | 3.7a | 2b | 10b | 38b | 9b |
| Abbott 6 percent | 12.2a | 1.9c | 2.1b | 1.2a | 3.3a | 2b | 9b | 33b | 7b |
| Abbott 3 percent | 12.3a | 2.1ab | 2.2ab | 1.3a | 3.5a | <1b | 7b | 8c | <1c |
| Control | 11.5a | 2.0bc | 1.7c | 1.3a | 3.0a | 0c | 8b | 0d | 0d |
| IMRD: Hillson Roottrainers®, loblolly pine | | | | | | | | | |
| IMRD 6 percent | 10.9b | 1.9b | 2.3b | 1.4a | 3.7b | 36a | 44a | 98a | 83a |
| Abbott 12 percent | 13.3a | 2.2a | 3.0a | 1.4a | 4.4a | 1b | 6b | 33b | 6b |
| Abbott 6 percent | 11.6b | 1.9b | 2.5b | 1.3a | 3.8b | 1b | 6b | 18c | 3b |
| Abbott 3 percent | 10.8b | 2.2a | 2.3b | 1.3a | 3.6b | <1b | 6b | 5d | <1c |
| Control | 11.5b | 2.0b | 2.2b | 1.3a | 3.6b | 0c | 8b | 0e | 0d |
| Louisiana:Styroblock-4, longleaf pine | | | | | | | | | |
| IMRD 6 percent | — | 3.0a | 2.5a | 1.3ab | 3.8b | 16a | 34a | 98a | 44a |
| Abbott 12 percent | — | 2.9a | 2.8a | 1.2b | 4.0ab | 9ab | 30ab | 85a | 26b |
| Abbott 6 percent | — | 3.1a | 2.8a | 1.4a | 4.2a | 6b | 34a | 83a | 15bc |
| Abbott 3 percent | — | 2.8a | 2.6a | 1.2b | 3.8b | 4b | 34a | 60b | 7c |
| Control | — | 3.1a | 2.5a | 1.4a | 3.9ab | 0c | 26b | 0c | 0d |
| Louisiana:Styroblock-4, shortleaf pine | | | | | | | | | |
| IMRD 6 percent | 7.9b | 1.5b | 0.7c | 0.7b | 1.4c | 23a | 33a | 100a | 69a |
| Abbott 12 percent | 11.2a | 1.7a | 1.1a | 0.9a | 2.0a | 19ab | 29a | 100a | 65a |
| Abbott 6 percent | 10.8a | 1.6a | 1.0a | 0.9a | 1.9a | 13b | 30a | 100a | 43b |
| Abbott 3 percent | 9.9a | 1.7a | 0.9ab | 0.9a | 1.8ab | 6c | 32a | 65b | 13c |
| Control | 9.5ab | 1.5b | 0.8bc | 0.8ab | 1.6bc | 0d | 20b | 0c | 0d |
| Nebraska:Styroblocks- Colorado State, ponderosa pine | | | | | | | | | |
| IMRD 6 percent | 11.3c | 2.2c | 2.1d | 3.6a | 5.7c | 64a | 72a | 100a | 88a |
| Abbott 12 percent | 14.7a | 2.7a | 3.9a | 4.0a | 7.9a | 40b | 57b | 98ab | 69b |
| Abbott 6 percent | 12.5b | 2.5b | 3.0b | 4.1a | 7.1b | 30bc | 53bc | 93bc | 51b |
| Abbott 3 percent | 11.9bc | 2.3c | 2.6c | 3.7a | 6.3bc | 17c | 45c | 90c | 34c |
| Control | 11.2c | 2.2c | 2.4cd | 3.8a | 6.2c | 0d | 22d | 0d | 0d |
| Nebraska:Styroblocks- Colorado State, Scotch pine | | | | | | | | | |
| IMRD 6 percent | 7.8b | 1.7c | 1.5d | 2.8b | 4.3d | 61a | 66a | 100a | 92a |
| Abbott 12 percent | 9.9a | 2.2a | 3.2a | 5.4a | 8.6a | 39b | 52b | 100a | 74b |
| Abbott 6 percent | 8.0b | 2.0b | 2.2b | 4.6a | 6.8b | 43b | 58ab | 100a | 75b |
| Abbott 3 percent | 7.7b | 1.9c | 2.0c | 3.3b | 5.3c | 18c | 38c | 80b | 37c |
| Control | 6.9c | 1.8c | 1.6d | 3.1b | 4.7cd | 0d | 11d | 0c | 0d |

TABLE 2. Continued.

| Location, container, and species and Pt inoculum treatment | Height (cm) | Stem diam (mm) | Fresh weight (gm) | | | Percent short roots ectomycor- rhizal with— | | Percent of seed- lings with Pt ecto- mycor- rhizae | |
|---|----------------|----------------------|-------------------|-------|--------|--|--------------|--|------|
| | | | Top | Root | Total | Pt | All fungi | Pt index ² | |
| Nebraska:Styroblocks- Colorado State, Austrian pine | | | | | | | | | |
| IMRD 6 percent | 8.4ab | 2.1cd | 2.2cd | 2.8b | 5.0c | 64a | 75a | 100a | 85a |
| Abbott 12 percent | 8.9a | 2.5a | 3.7a | 4.3a | 8.0a | 35b | 55b | 100a | 63b |
| Abbott 6 percent | 8.9a | 2.3b | 2.8b | 3.9a | 6.7b | 33b | 53b | 98a | 62b |
| Abbott 3 percent | 8.5ab | 2.2bc | 2.4c | 3.0b | 5.4c | 35b | 52b | 100a | 67b |
| Control | 7.8b | 2.0d | 2.0d | 3.0b | 5.0c | 0c | 19c | 0c | 0c |
| North Dakota:Tinus Roottrainers®, bur oak | | | | | | | | | |
| IMRD 6 percent | 18.0bc | 4.9a | 2.4ab | 9.6a | 12.0ab | 43a | 47a | 100a | 92a |
| Abbott 12 percent | 20.2ab | 4.6a | 2.4ab | 9.6a | 12.0ab | 46a | 51a | 100a | 90a |
| Abbott 6 percent | 21.5a | 5.1a | 2.6a | 10.1a | 12.7a | 38ab | 44a | 100a | 86a |
| Abbott 3 percent | 19.9ab | 4.4a | 2.1ab | 9.4a | 11.5ab | 29b | 36b | 100a | 80a |
| Control | 17.0c | 4.3a | 1.7c | 8.2a | 9.9b | 0c | 9c | 0b | 0b |
| Oregon:Leach tubes, Douglas-fir | | | | | | | | | |
| IMRD 6 percent | 16.1a | 2.0b | 1.7c | 3.5c | 5.2c | 41a | 44a | 100a | 93a |
| Abbott 12 percent | 16.9a | 2.2a | 2.1a | 5.0a | 7.1a | 15b | 17bc | 83ab | 73b |
| Abbott 6 percent | 16.7a | 2.1b | 2.0ab | 3.7bc | 5.7b | 17b | 22b | 60b | 46bc |
| Abbott 3 percent | 15.4a | 2.1b | 1.9ab | 3.9b | 5.8b | 8b | 14bc | 58b | 33c |
| Control | 15.8a | 2.1b | 1.8bc | 3.4c | 5.2c | 0c | 8c | 0c | 0d |
| Oregon:Leach tubes, western hemlock | | | | | | | | | |
| IMRD 6 percent | 14.7ab | 2.0ab | 2.0ab | 2.3a | 4.3ab | 15a | 18a | 85a | 71a |
| Abbott 12 percent | 16.1a | 2.1a | 2.1ab | 2.5a | 4.6a | 12a | 17a | 80a | 56b |
| Abbott 6 percent | 15.8a | 2.1a | 2.2a | 2.3a | 4.5a | 12a | 14a | 73a | 63ab |
| Abbott 3 percent | 15.9a | 2.0ab | 2.2a | 2.2ab | 4.4ab | 6b | 9b | 40b | 27c |
| Control | 13.6b | 1.9b | 1.7b | 2.0b | 3.7b | 0c | 5b | 0c | 0d |
| Canada:Ferdinand Roottrainers®, jack pine—high fertility | | | | | | | | | |
| IMRD 6 percent | 12.6a | 1.6a | 1.9a | 1.4a | 3.3a | 8a | 10a | 60a | 43a |
| Abbott 12 percent | 12.6a | 1.8a | 1.6ab | 1.4a | 3.0ab | 7a | 10a | 55a | 38a |
| Abbott 6 percent | 12.0ab | 1.5a | 1.8a | 1.2ab | 3.0ab | 2b | 5b | 30b | 10b |
| Abbott 3 percent | 12.4a | 1.4a | 1.4b | 0.9b | 2.3b | 1b | 3b | 8c | 2c |
| Control | 11.1b | 1.4a | 1.5ab | 1.1ab | 2.6b | 0c | 2b | 0d | 0d |
| Canada:Ferdinand Roottrainers®, jack pine—low fertility | | | | | | | | | |
| IMRD 6 percent | 8.2a | 1.3a | 1.0a | 1.1a | 2.1a | 36a | 39a | 85a | 78a |
| Abbott 12 percent | 8.3a | 1.4a | 1.0a | 1.2a | 2.2a | 15b | 18b | 73a | 60a |
| Abbott 6 percent | 8.3a | 1.3a | 0.9a | 0.8b | 1.7ab | 8c | 10b | 38b | 26b |
| Abbott 3 percent | 6.7ab | 1.2a | 0.7ab | 0.8b | 1.5ab | 1d | 4c | 10c | 4c |
| Control | 5.9b | 1.1a | 0.5b | 0.6b | 1.1b | 0e | 6c | 0d | 0d |

¹ Means sharing a common letter within a location-tree species combination but between Pt inoculum treatments are not significantly different at $P = 0.05$.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

mycorrhizal development by *P. tinctorius*. Regardless of inoculum source or rate, the Pt indices at high-fertility levels were about half those at the low-fertility level. As expected, however, growth of the jack pine seedlings was much better at the higher fertility level.

Although the 1978 Abbott inoculum was considerably more effective than that produced by Abbott in 1977, it was still not as effective as the IMRD inoculum in most tests. The 1978 Abbott inoculum also stimulated seedling growth in certain tests, as it did in 1977. The only test in which increased *P. tinctorius* ectomycorrhizal development could be related to increased seedling growth, however, was in Canada with jack pine at both fertility levels. Even though the 1978 Abbott inoculum contained abundant mycelium of *P. tinctorius* which could be detected in the vermiculite particles with the unaided eye, it was suspected to contain more nutrients and microbial contaminants that could have decreased the effectiveness of the inoculum.

As in the 1977 tests, the prevalence of naturally occurring ectomycorrhizal fungi was greater in the southern test areas than in the northern ones. Regardless of test location, most of these ectomycorrhizae morphologically resembled those of *Thelephora terrestris*. Sporophores of this fungus occurred on the bottoms of several control seedling containers in Nebraska. Seedlings in North Dakota had a high incidence of a white, coralloid ectomycorrhizae as in the 1977 tests; this type also occurred to a lesser extent on seedlings in Nebraska, Canada, and Oregon. Seedlings with abundant *P. tinctorius* ectomycorrhizae had few naturally occurring ectomycorrhizae which indicated that the introduced inoculum competed well against natural sources of inoculum.

Overall, the most consistent development of *P. tinctorius* ectomycorrhizae from both inoculum sources was obtained with bur oak in North Dakota. The probable reasons were the larger volume of growing medium used for each seedling, better cultural conditions (light, CO₂, etc.), the low incidence of naturally occurring ectomycorrhizal fungi, and the long growth phase of the test. A small amount of viable inoculum in large containers of growing medium may be as effective after 6 mo as a larger amount of inoculum in a smaller container of growing medium for a shorter period if there are few other fungi competing for the same feeder roots.

Results from the 1978 tests indicate that viable inoculum of *P. tinctorius* in vermiculite and peat moss can be produced in industrial fermentors. Some batches were nearly as effective as IMRD inoculum, however, other batches of Abbott inoculum produced using 1978 procedures and tested in various bare-root nurseries in the United States in the spring of 1978 and 1979 varied significantly in their ability to form *P. tinctorius* ectomycorrhizae (Marx, unpublished data). Further modifications in fermentation procedures and quality control at Abbott Laboratories appeared necessary to produce effective inoculum consistently.

1979 AND 1980 TESTS

INOCULUM CHARACTERIZATIONS

Since lack of consistent inoculum quality was identified as a problem, we tried in 1979 and 1980 to develop some reliable methods for rapidly assessing the effectiveness of inoculum batches. In the past, the fastest method was to test the inoculum in a container study using a fast growing tree host such as loblolly pine. These container-grown seedling tests took far too long—a minimum of 12 to 14 wks.

During the fall of 1979 and winter of 1980, 12 batches of different formulations of inoculum were produced by Abbott and tested at the IMRD by various fast assay techniques for ectomycorrhizal synthesis. Nonmycorrhizal roots of loblolly pine seedlings grown in plastic pouches (Fortin and others 1980) were inoculated. An inoculum slurry-root dip technique described by Marx and others (1977b) was

also tested. Both techniques utilize pine seedlings with abundant nonmycorrhizal roots developed several weeks prior to inoculation. Although growing nonmycorrhizal seedlings for the plastic pouch technique was time consuming, when seedlings were inoculated and incubated properly with effective inoculum, abundant *P. tinctorius* ectomycorrhizae were formed in 3 to 4 wks. The second technique proved to be simpler and furnished more consistent results. The latter technique was used to assay subsequent inoculum batches. Results of these fast assays were used to direct changes in fermentation procedures at Abbott Laboratories as discussed later.

The inoculum slurry-root dip fast assay technique was as follows. Loblolly pine seed were sown in flats containing autoclaved vermiculite and grown in the mycorrhizal fungus-free growth room at the IMRD (Marx 1973) with 75 percent of full sunlight for 10 to 11 hrs daily. Seedlings were fertilized once after 5 wks growth with the equivalent of 5 ml/seedling of a 2,000 mg/l solution of water-soluble NPK fertilizer (20-19-18). Ten wks after germination, seedlings were carefully removed and their roots rinsed free of growing medium. Seedlings with at least three lateral roots 4 cm long, each supporting at least 10 short roots, were selected. Primary roots were trimmed to 8 cm. Roots of individual seedlings were totally immersed in a well-mixed, 1:1 slurry of inoculum and water for 5 sec. Seedlings were then allowed to drain for a few sec, and transplanted into cavities of Hillson Rootainers® containing a 1:1 vermiculite and peat moss rooting medium. The containers were placed on a greenhouse bench with 12 hrs of full sunlight supplemented with 4 hrs daily of approximately 8,000 lx of incandescent light. Seedlings were watered three times a wk and were not fertilized. Each batch of inoculum and control inoculum (1:1 vermiculite and peat moss) were replicated five times with eight seedlings per replicate in a randomized block design. After 25 days of growth, seedlings were removed from the containers and their roots rinsed free of growing medium. Roots were visually assessed for ectomycorrhizal development.

During the 1979-80 testing of various Abbott inocula, certain characteristics of the inoculum were determined shortly after drying and before seedling tests. The purpose of these characterizations was to find a reliable early indicator which would correlate with the effectiveness of the inoculum. One promising indicator was a propagule count of *P. tinctorius* in inoculum. It was accomplished by spreading 1 cc of dried inoculum onto petri plates containing 10 ml of MMN agar medium fortified with 25 mg/l benomyl and 10 mg/l of erythromycin phosphate. The plates (10 per inoculum batch) were incubated at 30°C for 5 days, growth centers of the yellow-gold mycelium of *P. tinctorius* were counted, and counts were expressed as propagules Pt/g of inoculum. Since inoculum particle size was suspected to affect survival of Pt mycelium another assay was developed to determine the amount of Pt in the smaller particles. This was done by screening inoculum through a No. 6 sieve, placing 30 or 40 of the particles that collected on a No. 8 sieve on the surface of fortified MMN agar medium and incubating as previously described. The percent of these particles yielding mycelial growth of *P. tinctorius* was recorded as percent Pt from small particles. Microbial contamination was determined by blending 1 g of inoculum with 100 ml of sterile water for 3 min, serially diluting this blend by factors of 10, and plating 1 ml plates of Difco trypticase soy agar. Plates were incubated for 5 days at 30°C, and bacterial and fungal colonies were counted. The dilution yielding less than 10 colonies of bacteria or fungi per plate was considered the contamination level. To determine pH, inoculum was mixed with distilled water in a 1:1 volume ratio and after 30 min, pH was measured with a glass electrode. Since glucose can function as a carbon source for undesired contaminating microorganisms during inoculum storage and in soil (Marx 1980), residual glucose (mg glucose/g of oven-dried inoculum)

in the inoculum was determined with an autoanalyser by a modification of Hoffman's (1937) technique. Tests in 1979–80 with the 12 batches of Abbott inoculum showed that 60 percent of the glucose in inoculum fresh from the fermentor was removed by leaching with water. Leached inoculum with low residual glucose was found to be more effective in the above fast assay technique than unleached inoculum containing more residual glucose. Attempts were made to correlate inoculum characteristics and results of fast assay with results of the 1980 container tests.

Since effectiveness of different batches of Abbott inoculum varied in bare-root nursery tests in 1978 and 1979 (Marx, unpublished data), five different batches of improved Abbott inoculum were tested in containers in 1980. Another variable, captan, was examined also as a suppressant of other microorganisms. Various fungicides were reported recently to affect *P. tinctorius* ectomycorrhizal development on container-grown pine seedlings (Pawuk and others 1980) and on pine seedlings grown in fumigated nursery soil (Marx and Rowan 1981). These authors suggested that certain fungicides, such as captan and benomyl, enhanced the efficacy of the IMRD inoculum by depressing populations of other microorganisms which are either present in inoculum or colonize soil after inoculum has been added. Repeated applications of captan were reported to depress development of *P. tinctorius* ectomycorrhizae on container-grown longleaf pine seedlings (Pawuk and others 1980), but we thought that a single initial application at seeding might provide suitable microbial control without affecting the inoculum. Therefore, captan was a treatment variable in the 1980 tests.

MATERIALS AND METHODS

IMRD inoculum was produced as in 1977 and 1978. Three different batches of inoculum were produced by Abbott Laboratories at weekly intervals for the initial 1980 container tests. These were produced by solid-substrate fermentation with vermiculite containing 5 to 10 percent peat moss by volume and routine nutrients. After steaming, the substrate was inoculated with twice as much starter mycelium of *P. tinctorius* (produced as in 1977 and 1978) as was used in the earlier tests and incubated. After incubation, the inoculum was leached with water to remove nutrients and then dried.

A fourth and fifth batch of Abbott inoculum were produced by similar procedures, but a newly constructed solid-substrate fermentor was used which had the facility to autoclave the substrate. In it, the vermiculite-peat moss-nutrient substrate was autoclaved prior to addition of starter mycelium. Inoculation, incubation, leaching, harvesting, and drying were the same as for batches 1, 2, and 3. Batches 4 and 5 were tested in separate container experiments.

The initial inoculum characteristics mentioned earlier were measured at Abbott Laboratories. Abbott inoculum was assayed within 1 day after drying and the IMRD inoculum was assayed 10 days after drying at the IMRD. All other assessments, including the fast assay, were done at the IMRD. Before being tested in the inoculum slurry-root dip fast assay, IMRD inoculum and Abbott batches 1, 2, 3, 4, and 5 were stored at 5°C for 28, 28, 21, 14, 8, and 5 days, respectively.

The initial 1980 container seedling test was done at the IMRD using Abbott batches 1, 2, and 3, an equal mixture of the three batches, IMRD inoculum, and a control (pure vermiculite) treatment. Each inoculum, mixed 10 percent by volume with 1:1 vermiculite and peat moss rooting medium, was placed in Hillson Rootainers®. IMRD inoculum and Abbott inoculum batches 1, 2, and 3 were stored at 5°C for 23, 23, 16, and 9 days, respectively, prior to use. Stratified loblolly pine seeds were planted in each of the 32 cavities per tray and five replicate trays were prepared for each of the six main treatments. Twenty-five

ml of a captan suspension (55 mg of 50 percent wettable powder/l water) were applied as a drench in each of 16 cavities per tray immediately after seeding to provide a dose of captan equivalent to 4.5 kg of active ingredient/ha. Seedlings were thinned to one per cavity 2 wks later. Twenty-five ml of a modified nutrient solution (Marx and others 1977b) containing 200 and 20 mg/l of N and P, respectively, were applied to each seedling 4 and 8 wks after germination. Seedlings were grown under conditions similar to those in the fast assay. The study was terminated after 14 wks, seedlings were assessed, and data were evaluated by split-plot analyses.

A test was conducted with Abbott inoculum batch 4, IMRD inoculum, and pure vermiculite control to determine the effect of inoculum rate and captan on *P. tinctorius* ectomycorrhizal development and growth of loblolly pine seedlings. IMRD and Abbott inocula were stored for 29 and 9 days, respectively, prior to use. Both inoculum sources and the vermiculite control were mixed at 10, 5, 3.3, and 2.5 percent by volume with a 1:1 volume mixture of the vermiculite and peat moss rooting medium. Five trays of Hillson Rootainers® were filled with each, seeded, and half of each tray was drenched with captan as described earlier. Other procedures, growing conditions, and analyses were the same as in the initial 1980 container test.

Abbott inoculum batch 5, in comparison with IMRD inoculum and pure vermiculite control, was tested as had been batches 1, 2, and 3. IMRD and Abbott inocula were stored at 5°C for 36 and 13 days, respectively, prior to use.

RESULTS AND DISCUSSION

The IMRD inoculum and the five batches of Abbott inocula varied considerably in their characteristics (Table 3). The most striking difference between the two sources was that the IMRD inoculum produced only two colonies of *P. tinctorius* in one test and no colonies in another test after plating on agar medium, whereas Abbott inocula produced numerous colonies of *P. tinctorius* in both plating tests. Residual glucose in the Abbott inocula varied from 3 times more to 3 times less than that detected in the IMRD inoculum. Abbott inoculum batches 3, 4, and 5 and the IMRD inoculum contained bacterial and fungal contaminants, but batches

TABLE 3. Characteristics of inoculum of *Pisolithus tinctorius* (Pt) produced in 1980 by the Institute for Mycorrhizal Research and Development (IMRD) and Abbott Laboratories.¹

| Inoculum | Bulk density (g/l) | Moisture content (percent) | Propagules Pt (Pt/g) | Pt from small particles (percent) | Residual glucose (mg/g) | pH | Contaminants | |
|----------|--------------------|----------------------------|----------------------|-----------------------------------|-------------------------|------------------|-------------------|-----------------|
| | | | | | | | Bacteria dilution | Fungi dilution |
| IMRD | 350 | 38 | 2 | 0 | 5.7 | 4.6 | 1×10^5 | 1×10^5 |
| ABBOTT | | | | | | | | |
| Batch 1 | 300 | 27 | 40 | 92 | 15.8 | 5.4 ² | 1×10^5 | 0 |
| Batch 2 | 247 | 28 | 41 | 53 | 7.2 | 5.6 ² | 1×10^5 | 0 |
| Batch 3 | 268 | 26 | 4 | 70 | 13.9 | 5.1 ³ | 1×10^6 | 3×10^5 |
| Batch 4 | 212 | 23 | 41 | 81 | 3.6 | 5.1 ³ | 1×10^4 | 1×10^4 |
| Batch 5 | 357 | 19 | 19 | 43 | 1.8 | 5.5 ³ | 1×10^4 | 1×10^4 |

¹ Details of methods used to obtain characteristics are presented in text.

² 5 percent peat moss by volume with vermiculite in fermentor.

³ 10 percent peat moss by volume with vermiculite in fermentor.

TABLE 4. Ectomycorrhizal development of *Pisolithus tinctorius* (Pt) on loblolly pine seedlings by various batches of 1980 inoculum produced by the Institute for Mycorrhizal Research and Development (IMRD) and Abbott Laboratories using the inoculum slurry-root dip, fast assay technique. Each value is the mean from 8 seedlings per treatment in each of 5 blocks.¹

| Inoculum source | Percent of seedlings with Pt ectomycorrhizae | Percent short roots ectomycorrhizal with— | | Pt index ² |
|-----------------|--|---|-----------|-----------------------|
| | | Pt | All fungi | |
| IMRD | 100 | 68 | 70 | 97 |
| Abbott #1 | 100 | 74 | 75 | 99 |
| Abbott #2 | 100 | 63 | 65 | 97 |
| Abbott #3 | 100 | 53 | 55 | 95 |
| Abbott #4 | 100 | 59 | 62 | 95 |
| Abbott #5 | 100 | 56 | 60 | 93 |
| Control | 0 | 0 | 3 | 0 |

¹ There were no significant differences between means in columns obtained from IMRD and Abbott inocula.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

1 and 2 of Abbott's inoculum contained only bacteria. Abbott batch 3 contained more bacterial and fungal contaminants than other inocula. Most microbial contamination probably occurred during the drying process. Abbott inocula containing 10 percent peat moss were slightly more acid (pH 5.3) than inocula containing 5 percent peat moss (pH 5.5), but all were less acid than the IMRD inoculum (pH 4.6) which contained only 3.4 percent peat moss. There was apparently no relationship between residual glucose and microbial contamination. Abbott inoculum batches 4 and 5, produced in the new fermentor which sterilized the growing medium, contained fewer microbial contaminants than Abbott batches 1, 2, and 3 produced after only steaming of the growing medium.

In the root dip fast assay, the five batches of Abbott inocula were as effective in forming *P. tinctorius* ectomycorrhizae as the IMRD inoculum (Table 4). Thus, under the test conditions, sufficient quantities of viable hyphae of *P. tinctorius* were present in all inoculum sources to infect roots and form typical *P. tinctorius* ectomycorrhizae.

In the test of Abbott batches 1, 2, and 3, captan had no significant effect on seedling growth and only slight negative influence on ectomycorrhizal development in one batch of inoculum (Abbott batch 3, Table 5). Batch 3 also formed significantly fewer *P. tinctorius* and had a lower Pt index than did the IMRD inoculum. Abbott batch 3 had more contaminating microorganisms than other inocula used in this test. The Pt indices of other Abbott inocula, including the batch mixture, with or without captan did not differ from that of IMRD inoculum. All batches, including batch 3, had Pt indices above 50, the minimum acceptable value for inoculum effectiveness. The mixed batch had a Pt index as good as that of batches 1 and 2, indicating that highly effective Abbott inoculum can be diluted by at least a third with less effective inoculum and not lose detectable effectiveness. There were no differences in seedling growth due to *P. tinctorius* ectomycorrhizal development and generally the control seedlings were larger than inoculated seedlings. About 40 percent of all captan-treated, control (noninocu-

TABLE 5. Results of 1980 container-grown loblolly pine seedling test with vegetative inoculum of *Pisolithus tinctorius* (Pt) produced by the Institute for Mycorrhizal Research and Development (IMRD) and three batches produced by Abbott Laboratories interacting with a captan drench. Each value is the mean of 10 seedlings from each of 5 blocks.¹

| Inoculum and captan treatment | Height (cm) | Stem diam (mm) | Fresh weight (gm) | | | Percent short roots ectomycorrhizal with— | | Percent of seedlings with Pt ectomycorrhizae | Pt index ² |
|-------------------------------|-------------|----------------|-------------------|------|-------|---|-----------|--|-----------------------|
| | | | Top | Root | Total | Pt | All fungi | | |
| IMRD inoculum | | | | | | | | | |
| None | 14.4b | 1.7b | 1.4b | 1.1a | 2.5b | 53a | 59a | 100a | 90a |
| Captan | 13.8b | 1.8ab | 1.5b | 1.3a | 2.8b | 58a | 63a | 100a | 91a |
| Abbott inoculum—batch 1 | | | | | | | | | |
| None | 14.4b | 1.8b | 1.4b | 1.1a | 2.5b | 45ab | 53ab | 100a | 83ab |
| Captan | 15.3ab | 1.8b | 1.5ab | 1.2a | 2.7b | 39b | 47b | 100a | 83ab |
| Abbott inoculum—batch 2 | | | | | | | | | |
| None | 14.4b | 1.7b | 1.4b | 1.1a | 2.5b | 38b | 45bc | 100a | 83ab |
| Captan | 15.0ab | 1.8ab | 1.6ab | 1.2a | 2.6b | 37b | 45b | 100a | 82ab |
| Abbott inoculum—batch 3 | | | | | | | | | |
| None | 15.1b | 1.8b | 1.5b | 1.2a | 2.7ab | 33b | 41c | 100a | 79b |
| Captan | 15.3ab | 1.8ab | 1.5ab | 1.4a | 2.9ab | 26c | 35c | 96b | 70b |
| Abbott inoculum—mixed batches | | | | | | | | | |
| None | 14.4b | 1.8b | 1.5b | 1.2a | 2.7ab | 42ab | 49abc | 100a | 83ab |
| Captan | 15.1ab | 1.7b | 1.6ab | 1.3a | 2.9ab | 35b | 43bc | 100a | 81ab |
| Control | | | | | | | | | |
| None | 16.7a | 1.9a | 1.7a | 1.2a | 2.9a | 0d | 16d | 0c | 0c |
| Captan | 16.0a | 1.9a | 1.7a | 1.3a | 3.1a | 0d | 19d | 0c | 0c |

¹ Numbers in a column followed by the same letter are not significantly different at $P = 0.05$.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

lated) seedlings had *T. terrestris* sporophores as compared to 10 to 18 percent of seedlings from the other treatments.

The test of Abbott inoculum batch 4 showed that captan generally depressed seedling growth but increased Pt indices, especially at the three lowest rates of Abbott inoculum and the lowest rate of IMRD inoculum (Table 6). Pt indices decreased as rate of inoculum decreased, regardless of captan treatment. Captan increased the average Pt index for all rates of Abbott inoculum by over 70 percent, and for all rates of IMRD inoculum by 8 percent. Among the Abbott inoculum-fungicide treatments, only the 5 percent inoculum rate with captan produced a Pt index above 50. Conversely, among the IMRD inoculum-fungicide treatments, only the lowest inoculum rate without captan produced a Pt index less than 50. This test showed that effective inoculum can be diluted with as many as 39 parts of rooting medium and still produce an acceptable amount of *P. tinctorius* ectomycorrhizae. In no treatment did *T. terrestris* sporophores occur on more than 12 percent of test seedlings.

In the test of Abbott inoculum batch 5, captan had no effect on *P. tinctorius*

TABLE 6. Results of 1980 container-grown loblolly pine seedling test with different rates of vegetative inoculum of *Pisolithus tinctorius* (Pt) produced by the Institute for Mycorrhizal Research and Development (IMRD) and batch 4 produced by Abbott Laboratories interacting with a Captan drench. Each value is the mean of 10 seedlings from each of 5 blocks.¹

| Inoculum rate and captan treatment | Height (cm) | Stem diam (mm) | Fresh weight (gm) | | | Percent short roots ectomycorrhizal with— | | Percent of seedlings with Pt ectomycorrhizae | Pt index ² |
|------------------------------------|-------------|----------------|-------------------|-------|-------|---|-----------|--|-----------------------|
| | | | Top | Root | Total | Pt | All fungi | | |
| IMRD inoculum, 10 percent rate | | | | | | | | | |
| None | 16.7ab | 2.1a | 2.1ab | 1.5bc | 3.6ab | 54a | 66ab | 100a | 91a |
| Captan | 17.0ab | 2.1a | 2.2a | 1.7ab | 3.9a | 57a | 74a | 100a | 90a |
| Abbott inoculum, 10 percent rate | | | | | | | | | |
| None | 17.5a | 2.1a | 2.1ab | 1.6bc | 3.7ab | 14d | 37d | 96ab | 37d |
| Captan | 15.9bc | 1.9ab | 1.9bc | 1.5bc | 3.4bc | 21c | 39d | 94b | 48d |
| IMRD inoculum, 5 percent rate | | | | | | | | | |
| None | 17.0ab | 2.0ab | 2.1ab | 1.6bc | 3.7ab | 43ab | 52bc | 100a | 81b |
| Captan | 14.7c | 2.0ab | 1.9bc | 1.4c | 3.3bc | 47ab | 54b | 100a | 86ab |
| Abbott inoculum, 5 percent rate | | | | | | | | | |
| None | 17.7a | 2.1a | 2.3a | 1.6ba | 3.9a | 16d | 51b | 78c | 24ef |
| Captan | 16.2b | 2.0ab | 2.0b | 1.8a | 3.8a | 32b | 53b | 98ab | 57cd |
| IMRD inoculum, 3.3 percent rate | | | | | | | | | |
| None | 15.8bc | 1.9ab | 2.1ab | 1.4c | 3.6ab | 30bc | 43cd | 100a | 68c |
| Captan | 14.5c | 1.8b | 1.9bc | 1.3c | 3.2bc | 32b | 43cd | 96ab | 70bc |
| Abbott inoculum, 3.3 percent rate | | | | | | | | | |
| None | 16.8ab | 2.0ab | 2.0b | 1.4c | 3.4bc | 17d | 50bc | 70cd | 27ef |
| Captan | 14.8c | 1.9ab | 1.7c | 1.2c | 2.9c | 20cd | 43cd | 78c | 38d |
| IMRD inoculum, 2.5 percent rate | | | | | | | | | |
| None | 16.4b | 2.1a | 2.1ab | 1.5bc | 3.6ab | 24c | 42cd | 88bc | 49d |
| Captan | 15.7bc | 2.0a | 2.0b | 1.5bc | 3.5ab | 33b | 48c | 98ab | 69c |
| Abbott inoculum, 2.5 percent rate | | | | | | | | | |
| None | 17.1ab | 1.9ab | 2.0b | 1.6bc | 3.6ab | 13d | 48c | 66d | 19f |
| Captan | 16.5b | 1.9ab | 2.1ab | 1.5bc | 3.6ab | 20cd | 48c | 72c | 33d |
| Control | | | | | | | | | |
| None | 16.0bc | 2.0a | 1.8bc | 1.3c | 3.1bc | 0e | 37d | 0e | 0g |
| Captan | 15.1bc | 1.9ab | 1.7c | 1.2c | 2.9c | 0e | 40d | 0e | 0g |
| Overall effects | | | | | | | | | |
| None | 16.8* | 2.0 | 2.1* | 1.5 | 3.6 | 26 | 47 | 87 | 50 |
| Captan | 15.6 | 1.9 | 1.9 | 1.5 | 3.4 | 33* | 49 | 92 | 61* |

¹ Numbers in a column followed by the same letter are not significantly different at $P = 0.05$. * denotes significant difference between means of overall No captan and captan effects.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

TABLE 7. Results of 1980 container-grown loblolly pine seedling test with vegetative inoculum of *Pisolithus tinctorius* (Pt) produced by the Institute for Mycorrhizal Research and Development (IMRD) and batch 5 produced by Abbott Laboratories interacting with a captan drench. Each value is the means of 10 seedlings from each of 5 blocks.¹

| Inoculum and captan treatment | Height (cm) | Stem diam (mm) | Fresh weight (gm) | | | Percent short roots ectomy- corrhizal with— | | Percent of seed- lings with Pt ectomy- cor- rhizae | Pt index ² |
|-------------------------------------|----------------|----------------------|-------------------|-------|-------|--|--------------|--|--------------------------|
| | | | Top | Root | Total | Pt | All fungi | | |
| | | | | | | | | | |
| IMRD inoculum | | | | | | | | | |
| None | 16.6b | 2.1a | 2.3a | 2.0a | 4.3a | 53a | 60a | 100a | 87a |
| Captan | 18.6a | 2.0ab | 2.3a | 2.0a | 4.3a | 49a | 56a | 100a | 86a |
| Abbott inoculum, batch 5 | | | | | | | | | |
| None | 19.3a | 2.1a | 2.3a | 1.8ab | 4.1ab | 26b | 42b | 98a | 59b |
| Captan | 17.7ab | 1.9b | 2.1a | 1.7b | 3.8b | 24b | 41b | 94a | 54b |
| Control | | | | | | | | | |
| None | 18.8a | 2.0ab | 2.2a | 1.7b | 3.9ab | 0c | 37c | 0b | 0c |
| Captan | 18.4a | 2.0ab | 2.1a | 1.6b | 3.7b | 0c | 40c | 0b | 0c |

¹ Numbers in a column followed by the same letter are not significantly different at $P = 0.05$.

² Pt index = $a \times (b/c)$ where a = percent of seedlings with Pt ectomycorrhizae, $b = \bar{X}$ percent of feeder roots with Pt ectomycorrhizae (including 0 percent for those without Pt), and $c = \bar{X}$ percent of feeder roots with ectomycorrhizae formed by Pt and other fungi.

ectomycorrhizal development or growth of seedlings (Table 7). Seedlings in the IMRD inoculum treatment had more *P. tinctorius* ectomycorrhizae and greater Pt indices than seedlings from Abbott inoculum treatment. However, the Pt indices for Abbott inoculum batch 5, with or without captan, were greater than 50. Over 70 percent of the control seedlings, 36 percent of the seedlings in Abbott inoculum, and 16 percent of the seedlings in IMRD inoculum had *T. terrestris* sporophores. The occurrence of *T. terrestris* was not affected by captan.

There was very poor correlation between initial characteristics of inoculum or results of the fast assay and the results of the container-grown seedling tests. The two characterizations in which *P. tinctorius* was isolated from inoculum particles on agar medium both indicated high numbers of viable hyphae of *P. tinctorius* in all batches of Abbott inoculum. Most of these hyphae, however, were undoubtedly on the outside of the vermiculite particles. External hyphae would grow well on agar medium and also be capable of colonizing preformed short roots in the fast assay. However, in container seedling tests, external hyphae may fail to survive exposure in the growing medium for the 5 to 6 wks between seeding and short root development. IMRD inoculum, which was consistently effective in seedling tests, produced very few colonies of *P. tinctorius* on agar media. Apparently, the leaching and drying procedures used at the IMRD and the several days of inoculum storage before the characterization tests at Abbott destroyed most external hyphae of *P. tinctorius* on the vermiculite particles. These results and those obtained by direct microscopic examination of hyphae in the vermiculite particles suggest that more hyphae grew inside the laminated structures of the vermiculite in IMRD inoculum than in Abbott inoculum. These internal, protected hyphae are obviously responsible for inoculum effectiveness.

Microbial contamination appeared to inhibit inoculum effectiveness. Although some contamination was detected in all batches of inoculum, Abbott batch 3 had the most contaminants and was the least effective in forming ectomycorrhizae of *P. tinctorius*. Captan did not improve the effectiveness of this inoculum. The fungal contaminants may not have been inhibited by this fungicide, or the main inhibiting contaminants may have been bacteria which were not affected by captan. Certain bacteria can inhibit the effectiveness of inoculum of *P. tinctorius* (Bowen and Theodorou 1979). The effectiveness of Abbott batch 4 was strongly enhanced by captan, but batch 5 containing approximately the same amount of fungal and bacterial contaminants was not. Perhaps only certain microbes are being inhibited by captan, allowing a weak inoculum (batch 4) to become more effective but not influencing a more effective inoculum (batch 5) to the same extent. The amount of residual glucose in inoculum did not appear to be related to inoculum effectiveness in the container seedling tests. Abbott inoculum batch 1 had the highest residual glucose level, but it also produced higher Pt indices than other inocula with much lower glucose levels. Between pH 4.6 and 5.6, the effect of inoculum pH was also inconsistent. IMRD inoculum and Abbott inoculum batches 1 and 2 had pH 4.6, 5.4, and 5.6, respectively, and produced high Pt indices; Abbott batches 3 and 4 each had a pH of 5.1 and produced significantly lower Pt indices. The ranges of bulk density and moisture content in inoculum were not correlated with inoculum effectiveness either.

With the exception of Abbott inoculum batch 4, the first inoculum produced in the newly constructed fermentor, all 1980 Abbott batches produced Pt indices of 50 or more and were rated effective in forming *P. tinctorius* ectomycorrhizae in these container seedling tests.

CONCLUSIONS

The results of these tests on container-grown seedlings clearly show that viable vegetative inoculum of *Pisolithus tinctorius* in a vermiculite-peat moss substrate can be produced with industrial fermentation equipment and procedures for practical use in forestry. The Abbott Laboratories inoculum of *P. tinctorius* has the trademark MycoRhiz[®].¹ Although not all batches of MycoRhiz[®] were as effective in forming ectomycorrhizae of *P. tinctorius* as the IMRD inoculum, the improved formulation produced abundant ectomycorrhizae on the root systems of the test seedlings and produced Pt indices greater than 50. An index of 50 represents the minimum level of root colonization by *P. tinctorius* that has significantly increased survival and growth of southern pine seedlings planted on reforestation sites. It should be pointed out that a higher or lower Pt index may be necessary to improve field performance of other trees species or of southern pines planted on adverse sites.

Different batches of MycoRhiz[®] of varying effectiveness can be mixed, and the most effective batches appear to dominate the less effective ones, resulting in an acceptable Pt index. This is important since in all likelihood different batches will be mixed in commercial production.

It became apparent during testing in bare-root nurseries in 1978 and 1979 (unpublished data) and during the preliminary testing of the 12 different formulations of MycoRhiz[®] in the winter of 1979–80, that final pH of inoculum must be less than 6.0. Certain batches of inoculum produced in pure vermiculite had a final pH as high as 8.2. Although some test inoculum batches were mixed with citric acid buffer or acid peat moss after fermentation to lower the final pH below 6.0,

¹ Registered trademark Abbott Laboratories, North Chicago, Ill., for vegetative inoculum of *Pisolithus tinctorius*.

they were still ineffective in forming sufficient quantities of *P. tinctorius* ectomycorrhizae in the fast assay. In this test, inocula produced with acid peat moss (pH 4.0 to 5.0) as an initial component of the substrate in the fermentor had final pH of 5.0 to 5.8 and were highly effective in forming *P. tinctorius* ectomycorrhizae. The use of peat moss as an acidifying agent for vermiculite was originally developed because of failures in the permanent acidification of vermiculite with chemical buffers (Marx and Zak 1965). The ability of peat moss to lower and stabilize pH may not be the only benefit derived from using this component in production of *P. tinctorius* inoculum. Recently, it was reported that *P. tinctorius* produced fulvic and humic acids in pure culture (Tan and others 1978) and that vegetative growth of the fungus in pure culture was stimulated by fulvic acid from soil (Tan and Nopamornbodi 1979). Peat moss may be furnishing essential humic acids or their precursors to *P. tinctorius* much like organic matter does in forest soils. Humic acids or other compounds of peat moss may play a vital role not only in ectomycorrhizal development, but also in the production of effective inoculum of *P. tinctorius*. Recently, a hot water extract of peat moss was found to stimulate vegetative growth of *P. tinctorius* on MMN agar medium (Marx, unpublished data).

Results from earlier tests (Marx 1980), from tests with MycoRhiz[®] in 1977–80 on bare-root nursery seedlings, and from tests reported here indicate that the most effective inoculum is one with (1) abundant hyphae of *P. tinctorius* inside the vermiculite particles, (2) pH between 4.5 and 6.0, (3) the least amount of microbial contaminants, and (4) the lowest amount of residual glucose (effective leaching prior to drying).

A single application of captan as a drench at time of seeding did not reduce effectiveness of any inoculum and, in certain tests, it increased the effectiveness. Any treatment which may increase inoculum effectiveness should be considered as an asset to the practical use of MycoRhiz[®] in forestry.

In the container tests, growth of seedlings was seldom increased by ectomycorrhizae of *P. tinctorius*. This lack of correlation between juvenile growth and ectomycorrhizal development has been reported before (Marx and Barnett 1974, Ruehle and Marx 1977) and may be related to drain of photosynthate (sucrose) from the host by *P. tinctorius*. Harley (1973, 1978) stated that the carbohydrate drain is the normal expense paid by the tree host for the ectomycorrhizal association and that it may account for as much as 10 percent of the carbohydrates produced by the tree hosts. In all likelihood, seedlings with few ectomycorrhizae are subject to little carbohydrate drain and in the presence of high quantities of readily available NPK are able to grow faster than those with abundant ectomycorrhizae. Our most obvious exception was with jack pine in Canada in 1978. There, seedlings with the most *P. tinctorius* ectomycorrhizae were larger than control seedlings with few ectomycorrhizae, even at unusually high levels of N, P, and K. Pine seedlings in the high-fertility treatment, however, had only about half as many *P. tinctorius* ectomycorrhizae as those in the lower fertility treatment, even though the former seedlings were nearly twice as large as the latter. The rapid utilization of photosynthate for plant growth in the high-fertility treatment may have reduced root concentration of sucrose sufficiently to decrease the susceptibility of short roots to infection by *P. tinctorius* as was reported earlier in loblolly pine (Marx and others 1977b). Even though young tree seedlings with abundant ectomycorrhizae may be the same size or even smaller than similarly grown seedlings with few or no ectomycorrhizae after their brief growth in containers, it is the performance of these seedlings in the field that is important. Field results, especially those from adverse sites as discussed earlier, support the contention that seedlings with abundant ectomycorrhizae survive and grow faster than similar size or even larger seedlings with few or no ectomycorrhizae.

Wide ranges of conditions, cultural practices, and tree species were tested in this research. Many inoculum characteristics—pH, glucose content, microbial contamination, propagules of *P. tinctorius*—ranged widely. Differences in containers, duration of inoculum storage, rooting media, seed treatments, fertility regimes, temperature, CO₂ concentration, and light conditions were experienced at different test locations, also there were varying amounts of other ectomycorrhizal fungi acting as competitors to introduced inoculum at most locations. Some inocula, however, performed well over these broad ranges of physical, chemical, and biological conditions. The significance of other factors—duration of inoculum storage under different conditions, amounts of major and minor elements most effective for each tree species \times *P. tinctorius* combination, positive and negative effects of pesticides, length of time to grow each tree species in containers for optimum *P. tinctorius* ectomycorrhizal development—must await future research efforts. Even though there are several unknown factors, this research shows that commercially produced inoculum of *P. tinctorius* can be used to form abundant ectomycorrhizae on container-grown seedlings. Field tests will eventually determine the benefits of such inoculation.

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